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VERIFICATION OF A TRANSLATION

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Date: October 26, 2001



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7/PATS

**Nucleic acid-antibody conjugate for delivering a
foreign nucleic acid into cells**

A1

The object of gene therapy is to correct a genetic defect by carrying out an intervention on the DNA. It may be carried out according to two different approaches: either as a correction of the genotype by repairing the genetic abnormality, or by correction of the phenotype by transplanting a normal version of the gene, thus making it possible to compensate the defective gene which is still present. Gene therapy applies to the treatment of both constitutional and acquired genetic diseases. Thus, a certain number of constitutional genetic diseases are candidates for gene therapy; mention may be made, inter alia, of cystic fibrosis, Duchenne myopathy or adenosine deaminase deficiency (Cournoyer *et al.*, 1991, "Gene transfer of adenosine deaminase into primitive human hemotopoetic progenitor cells", Human Gene Therapie, 2: 203). Gene therapy also applies to combatting acquired diseases, the candidate diseases of which are cancers and infectious and viral diseases (AIDS, hepatitis).

In cancer therapy, the first experiments carried out with tumor infiltrating lymphocytes (TILs: tumor infiltrating lymphocytes) demonstrated that cells could be armed with cytotoxic factors (TNF, tumor necrosis factor) (Rosenberg *et al.* 1990, "Gene transfer into humans: immunotherapy of patients with advanced melanoma using infiltrating lymphocytes modified by retroviral gene transduction" N. Eng. J. Med. 323: 570-578) or be doped with cytokines; thus, Golumbek and colleagues have obtained therapeutic success on mouse renal cancers treated with cells which produce interleukin 4 (Golumbek *et al.* 1991, Science 254; 713-716).

Gene therapy carried out on the somatic cells of an individual suffering from a genetic defect poses

multiple methodological problems, the repaired or transplanted gene having to be expressed normally in a regular manner, i.e. at the correct site, at the correct moment and in a normal amount suited to needs; 5 the correction or transplant having to be indefinitely stable.

Among the *ex vivo* somatic gene transfer strategies developed in order to attempt to specifically and 10 effectively target cells of interest, mention should be made of: (i) the strategies using physical methods, such as coprecipitation with calcium phosphate, electroporation, microinjection, protoplast fusion, biolistics or artificial vehicles such as liposomes and 15 receptor ligands for example; (ii) and those making use of viral vectors (retroviruses, adenoviruses, AAVs, HSVs) (Ragot *et al.*, 1993 *Nature* 361: 647-650). Among the *in vivo* somatic gene transfer strategies developed, mention may be made of cellular vehicles which have 20 received the gene *ex vivo* beforehand (hematopoietic stem cells, lymphocytes, hepatocytes, endothelial cells, epithelial cells), viral vectors, the intramuscular injection of naked DNA and artificial vehicles.

25 One of the current difficulties of gene therapy relates to the *in vivo* targeting of the cells to be modified. Currently, only a small number of viral or synthetic approaches have been developed; they essentially exploit ligand-receptor interactions (Michael and 30 Curiel, 1994, "Strategies to achieved targeted gene delivery via the receptor-mediated endocytosis pathway", *Gene Therapy* 1: 223).

Various approaches using a viral vector have thus been 35 experimented with. A first approach consists in bridging, via streptavidin, biotinylated antibodies directed against a target cell structure to antibodies, also biotinylated, directed against the structures of

the retroviral envelope and therefore associated with a retrovirus (Roux *et al.*, 1989, Proc. Natl. Acad. Sci. USA 86: 9079-9083). Once bound to cells, retroviral vectors are internalized by endocytosis and are capable of avoiding the lysosome-endosome system via a mechanism of transferring the endosome to the cytoplasm, thus avoiding degradation of the transfected DNA and allowing said DNA to enter the cell nucleus. This approach has revealed a lack of specificity of *in vivo* targeting due to retroviral vectors attaching nonspecifically to the cell surface. A second viral approach has been developed, which uses ectopic viruses modified so as to bear a chimeric envelope ligand-protein at their surface (Kasahara *et al.* 1987 "Receptor-mediated *in vitro* gene transformation by a soluble DNA carrier system" J. Biol. Chem. 262: 4429). Finally, mention should be made of the approach developed by Neda *et al.* (1991 "Chemical modification of an ecotropic murine leukemia virus results in redirection of its target cell specificity" J. Biol. Chem. 226: 14143).

Nonviral synthetic approaches have also been experimented with. They are carried out by forming a complex between a ligand capable of binding to the surface of the target cell, and the DNA to be transferred. Mention should be made, first of all, of the approaches using artificial vehicles such as antibody-coated liposomes (immunoliposomes); this type of approach has not, at the present time, proved satisfactory since the immunoliposomes exhibit nonspecific activity probably subsequent to the nonspecific attachment of the liposomes to cell membranes. It has also become apparent that the effectiveness of transfer of the gene contained in the liposomes remains modest, although it is presumed that degradation associated with endosomes is avoided by using liposomes. Alternative approaches using compounds

which retain the ability to interact specifically with cell surface receptors have been developed. Specifically, various receptors naturally present at the surface of cells have the property of internalizing
5 into the cell after attachment to their ligand; thus, transferrin, the receptor of which has a ubiquitous tissue distribution, has been the subject of many experiments (transferrinfection) (Zenke et al., 1990, Proc. Natl. Acad. Sci. USA, 87: 3655-3659). Another
10 alternative has consisted in targeting asialoglycoproteins present at the surface of hepatocytes (Wu et al., 1991, J. Biol. Chem. 266: 14338-14342). Finally, another technique, termed "antifection", developed by one of the inventors of the present invention (Hirsch
15 et al., "Antifection: a new method to targeted gene transfection" 1993, Transpl. Proc. 25: 138) has been described; this technique consists in preparing an antibody-DNA vector which is delivered to a selected cell population (US patent 5 428 132).

20 Besides the problems of targeting of the vector, another difficulty to be overcome in gene therapy experiments lies in the transfer, inside the cells, of the DNA to be transfected and the protection of this
25 DNA against nuclease activities of the lysosomal cellular compartments, in order to obtain sizable expression of the transgene.

Various approaches have been developed in order to
30 reply to this problem; it has thus been possible to obtain increased effectiveness of the expression of the transgene by using lysosomotropic agents such as chloroquine (Zenke et al., 1990, Proc. Natl. Acad. Sci. USA 87: 3655-3659; Luthman et al. 1983, Nucleic Acids
35 Res. 11: 1295); such agents decrease the lysosomal destruction of the DNA by increasing the pH of the endosomes and inhibiting the transfer of the internalized material to the lysosomes. Another

approach consists in using protein domains which have cellular translocation activity. Advantage is taken of the property of these domains in order to help the transfected nucleic acids escape from the endosomal vesicles, in order to increase the effectiveness of the transfer of nucleic acid to the nucleus (Fominaya and Wels, 1995, J. Biol. Chem. 271: 10560). International patent application WO 94/04696 describes a system for transferring nucleic acid, composed of a translocation domain originating from *Pseudomonas aeruginosa* exotoxin A; the effectiveness of transfection and the specificity of such a transfer system appear to be very low. Another international patent application, WO 96/13599, also describes a system for transferring nucleic acid, composed of a recombinant monomeric protein comprising various functional domains, including a translocation domain, derived from toxins, preferably bacterial toxins, such as exotoxin A.

The gene therapy strategies previously mentioned require laborious preparations or sophisticated material and may present a certain biological risk. At the current time, there is a need to develop a simple and effective system for transferring nucleic acids, which makes it possible to introduce, specifically in target cells, effectively expressed nucleic acids. In this respect, the antifection technique (US patent 5 428 132), based on the use of antibodies to target DNA sequences of interest into target cells, is extremely promising since antibodies constitute an extremely effective tool for directing the transfer vector toward a particular cell type, due to the high affinity and high specificity of antibodies; in addition, the multitude of available monoclonal and polyclonal antibodies directed against the many tumor or normal cell structures brings a real advantage to this technology. However, the antifection technique described in US patent 5 428 132, although it enables

effective targeting, does not make it possible to obtain sizable expression of the transfected transgene.

It is therefore the object of the present invention to
5 improve the level of expression of the transgene
transfected into the target cells using the antifection
technology. These improvements relate to the addition
of a translocation domain to the DNA-antibody complex,
and/or to the use of DNA-binding protein to
10 noncovalently couple DNA to the complex and/or to
increase the effectiveness of transfer, and/or to the
addition of a cleavable peptide. These improvements
make it possible, in a spectacular and unexpected
manner, to increase by 2- to 10-fold the level of
15 expression of the transgene in the target cell.

The present invention therefore relates to a conjugate
for transferring a nucleic acid molecule into a cell,
characterized in that it comprises a nucleic acid
20 molecule, a translocation domain and an antibody
specific for a surface antigen of said cell, such that
said conjugate is transfected effectively into said
cell.

25 According to a first embodiment of the invention (A),
the conjugate according to the invention is
characterized in that said nucleic acid molecule,
translocation domain and antibody are conjugated by
means of at least one bridging agent.

30 According to a preferred embodiment (A), the conjugate
is characterized in that it also comprises a peptide
which can be cleaved with at least one glycolytic
and/or proteolytic enzyme, said antibody being attached
35 to said translocation domain via said cleavable
peptide. In this conjugate, the antibody and said
cleavable peptide may be attached either (i) covalently
via a bridging agent preferably selected from the group

composed of benzoquinone, EDC and APDP; or (ii) to a molecule of the avidin type by means of a bridging agent, which may be identical or different, and which is preferably selected from the group composed of
5 biotin, benzoquinone, EDC and APDP. The translocation domain of this compound is attached to said cleavable peptide via a covalent chemical bond. The term "covalent chemical bond" is intended to denote preferably a bond of the peptide type; according to a
10 particular embodiment, the peptide corresponding to the translocation domain attached to the cleavable peptide is obtained by chemical synthesis.

In this embodiment, the translocation domain may be
15 attached to a nucleic acid molecule either:

i) by means of a bridging agent which is preferably APDP; according to this embodiment, an even more preferred embodiment of the conjugate of the invention is characterized in that said antibody is attached to
20 said cleavable peptide via a covalent bond by means of said bridging agent EDC, said cleavable peptide being attached to said translocation domain via a covalent bond by means of chemical attachment, said translocation domain being attached to said nucleic
25 acid via a covalent bond by means of said bridging agent APDP.

ii) via a nucleic acid-binding molecule, said nucleic acid-binding molecule being attached to said translocation domain via a covalent bond by means of a
30 bridging agent which is preferably APDP. According to this embodiment, an even more preferred embodiment consists in that said antibody is attached to said cleavable peptide via a covalent bond by means of said bridging agent EDC, said cleavable peptide being
35 attached to said translocation domain via a covalent bond by means of chemical attachment, said translocation domain being attached to said nucleic acid-binding molecule via a covalent bond by means of

said bridging agent APDP, said nucleic acid-binding molecule binding said nucleic acid via noncovalent attachment.

5 According to a second embodiment (B), the invention relates to a conjugate, characterized in that it also comprises a nucleic acid-binding molecule, such that said translocation domain, said antibody and said nucleic acid-binding molecule are attached to a
10 molecule of the avidin type by means of a bridging agent, which may be identical or different, said nucleic acid-binding molecule being bound to said nucleic acid molecule.

15 According to another embodiment (B), the invention relates to a conjugate, characterized in that it also comprises a nucleic acid-binding molecule and a peptide which can be cleaved with at least one glycolytic and/or proteolytic enzyme, such that said translocation
20 domain, said antibody and said cleavable peptide are attached to a molecule of the avidin type by means of a bridging agent, which may be identical or different, said nucleic acid-binding molecule being bound to said nucleic acid molecule, said nucleic acid-binding
25 molecule being attached to said cleavable peptide and bound to said nucleic acid molecule.

According to another aspect (C), the invention relates to a conjugate for transferring a nucleic acid molecule
30 into a cell, characterized in that it comprises a nucleic acid molecule, an antibody specific for a cell surface antigen and a nucleic acid-binding molecule, such that said conjugate is transfected effectively into said cell; this conjugate is characterized in that
35 said nucleic acid molecule, said antibody and said nucleic acid-binding molecule are attached to a molecule of the avidin type by means of a bridging agent, which may be identical or different, said

nucleic acid-binding molecule being bound to said nucleic acid molecule.

According to a preferred embodiment (C), the above
5 conjugate is characterized in that it also comprises a peptide which can be cleaved with at least one glycolytic and/or proteolytic enzyme, said antibody being attached to said nucleic acid-binding molecule via said cleavable peptide; in this conjugate, said
10 antibody and said cleavable peptide are attached either (i) covalently via a bridging agent preferably selected from the group composed of benzoquinone, EDC and APDP, or (ii) via a molecule of the avidin type by means of a bridging agent, which may be identical or different,
15 and which is preferably selected from the group composed of biotin, benzoquinone, EDC and APDP. In this conjugate, said cleavable peptide is attached to said nucleic acid-binding molecule by means of a bridging agent which is preferably APDP, said nucleic acid-
20 binding molecule binding said nucleic acid via noncovalent attachment.

According to one embodiment (D), the invention relates to a conjugate for transferring a nucleic acid molecule
25 into a cell, characterized in that it comprises a nucleic acid molecule, an antibody specific for a cell surface antigen and a peptide which can be cleaved with at least one glycolytic and/or proteolytic enzyme, such that said conjugate is transfected effectively into
30 said cell. In this conjugate, said antibody and said cleavable peptide are attached either (i) covalently via a bridging agent preferably selected from the group composed of benzoquinone, EDC and APDP, or (ii) to a molecule of the avidin type by means of a bridging
35 agent, which may be identical or different, preferably selected from the group composed of biotin, benzoquinone, EDC and APDP. In this conjugate, said cleavable peptide is attached to said nucleic acid

either (i) via a covalent bond by means of a bridging agent which is preferably APDP, or (ii) via a nucleic acid-binding molecule, said nucleic acid-binding molecule being attached to said cleavable peptide via a covalent bond by means of a bridging agent which is preferably APDP. According to a particularly preferred embodiment of the invention, said conjugate also comprises a translocation domain which is optionally attached covalently, by means of a bridging agent, to said nucleic acid molecule and/or to said nucleic acid-binding molecule. According to another embodiment, said translocation domain is present within the conjugate without being covalently attached thereto.

15 The term "cleavable peptide" is intended to denote a peptide comprising one or more sequences which can be cleaved with glycolytic and/or proteolytic enzymes, preferably endosomal and/or lysosomal enzymes, such as for example cathepsins and trypsin. According to a particular embodiment, the cleavable peptide of the invention comprises at least one cathepsin B site and/or one cathepsin D site. Preferably, the cleavable peptide comprises a cathepsin B site and a cathepsin D site, separated by at least one amino acid, preferably by at least two amino acids, such as for example glycine; the cleavable peptide of the invention has the sequence: $X_1-X_2-F-Y-G-G-F-R-$ in which G represents glycine, and X_1 and X_2 represent amino acids which allow the chemical bonding or attachment of the antibody, such as for example two lysines (K). F-Y represents the dipeptide composed of the amino acids phenylalanine-tyrosine, which can be cleaved with cathepsin D; this sequence may optionally be replaced with L-Y (leucine-tyrosine), Y-L (tyrosine-leucine) or F-F (phenylalanine-phenylalanine). FR represents the dipeptide composed of the amino acids phenylalanine-arginine which can be cleaved with cathepsin B.

The bridging agent makes it possible to attach chemically (covalently), electrostatically or noncovalently all or some of the components of the conjugate. Among bridging agents which can be used in
5 the present invention, mention should be made of benzoquinone, carbodiimide and, more particularly, EDC (1-ethyl-3[3-dimethylaminopropyl]carbodiimide hydrochloride), dimaleimide, dithiobisnitrobenzoic acid, (DTNB), N-succinimidyl-S-acetyl thioacetate (SATA),
10 bridging agents having one or more phenylazide groups which react with ultraviolet (UV) rays and preferably N-[-4-(azidosalicylamino)butyl]-3'-(2-pyridyldithio)-propionamide (APDP), N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), 6-hydrazinonicotimide (HYNIC)
15 and biotin; benzoquinone, EDC, APDP and biotin being the cleaving agents preferably used. The expression "molecule of the avidin type" is intended to denote all molecules which bind with high affinity to biotin, and preferably the tetravalent molecule avidin,
20 streptavidin, neutravidin.

According to a preferred embodiment of the invention, the conjugate described above according to the various
25 embodiments of the invention is characterized in that said bridging agent is selected from the group composed of benzoquinone, biotin, carbodiimides and bridging agents having at least one phenylazide group which reacts to ultraviolet (UV) radiation. According to a preferred embodiment, the bridging agent is selected
30 from the group composed of benzoquinone, biotin, EDC and APDP.

According to another preferred embodiment of the invention, the conjugate described above according to
35 the second embodiment (B) is characterized in that the bridging agent which attaches said translocation domain and said antibody to the molecule of the avidin type is biotin and the bridging agent which attaches said

nucleic acid-binding molecule to the molecule of the avidin type is benzoquinone. According to another preferred embodiment of the invention, the conjugate described above according to the second embodiment (B) is characterized in that the bridging agent which attaches said translocation domain, said antibody and said nucleic acid-binding molecule is biotin. According to a particular embodiment of the invention, the conjugate described above according to the second embodiment (B) is characterized in that the translocation domain and the nucleic acid-binding molecule form a fusion protein. The term "fusion protein" is intended to denote a protein which contains protein domains originating from different proteins and encoded by the same DNA molecule obtained by recombinant DNA technology. This fusion protein and the antibody are attached to a molecule of the avidin type by means of bridging agents, which are identical or different, said fusion protein being bound to said nucleic acid molecule via its nucleic acid-binding domain.

According to another preferred embodiment of the invention, the conjugate which is described above according to the second embodiment (B) and which comprises a cleavable peptide is characterized in that the bridging agent which attaches said translocation domain and said antibody to the molecule of the avidin type is biotin and the bridging agent which attaches said cleavable peptide to the molecule of the avidin type is benzoquinone. According to another preferred embodiment of the invention, the conjugate described above according to the second embodiment (B) is characterized in that the bridging agent which attaches said translocation domain, said antibody and said cleavable peptide is biotin.

According to another preferred embodiment, the conjugate described above according to another

embodiment (C) of the invention is characterized in that the bridging agent which attaches said antibody to the molecule of the avidin type is biotin and the bridging agent which attaches said nucleic acid-binding molecule to the molecule of the avidin type is benzoquinone. According to another preferred embodiment, the conjugate described above is characterized in that said bridging agent is biotin.

10 The conjugate according to the invention is characterized in that the nucleic acid molecule of the conjugate is chosen from single-stranded DNA, double-stranded DNA, single-stranded RNA, double-stranded RNA and an RNA/DNA hybrid. According to a preferred

15 embodiment, said nucleic acid molecule is double-stranded DNA or single-stranded RNA which encodes a protein product of interest which is expressed effectively in said cell. The protein products of interest are chosen from a group composed of

20 interleukins, cytokines, lymphokines, chemokines, growth factors, killer proteins, proteins which make it possible to lift chemoresistance and restriction enzymes; the interleukins, cytokines and lymphokines are chosen from a group preferably composed of the

25 interleukins Il-1, Il-2, Il-3, Il-4, Il-5, Il-6, Il-7, Il-8, Il-9, Il-10, Il-11, Il-12, Il-13, Il-14, Il-15, Il-16, Il-17 and Il-18, and the interferons α -IFN, β -IFN and γ -IFN; preferably, the protein product of interest is interleukin 2. The growth factors are

30 preferably colony stimulating factors (G-CSF, GM-CSF and M-CSF) and erythropoietin; mention should also be made of the growth factors which interact, by inhibiting them, with nuclear transcription factors such as NF-KB; these growth factors were the subject of

35 patent application FR 98/14858. The killer proteins are chosen from the group composed of kinases, and preferably thymidine kinase, and pro-apoptotic proteins; the term "pro-apoptotic proteins" is intended

to denote the proteins which are involved in apoptosis or promote apoptosis. Among the pro-apoptotic proteins, mention should be made of the proteins of the Bcl2 family, and more particularly the BIK (Bcl2-interacting protein), BAX (Oltvai *et al.* 1993, *Cell* 74: 609-619), BAK (Chittenden *et al.* 1995, *Nature* 374: 733-736; Kiefer *et al.* 1995, *Nature* 374: 736-739) and BID (BH3-interacting domain death agonist) (Wang *et al.* 1996, *Genes Dev.* 10: 2859-2869) proteins; preferably, the protein product of interest is the BAX protein. Among the pro-apoptotic proteins, mention should also be made of caspases, the AIF (apoptosis-inducing factor) protein (Susin *et al.* 1999, *Nature* 397: 441-446) and the proteins of the tumor necrosis factor (TNF) family, and more particularly TNF itself (Old 1985, *Science* 230: 630-632) and the FASL (FAS-ligand) protein (Takahashi *et al.*, 1994, *Int. Immun.* 6: 1567-1574).

According to another embodiment of the invention, the nucleic acid molecule is an antisense RNA.

According to the invention, the conjugate according to the invention is characterized in that the nucleic acid-binding molecule binds said nucleic acid molecule via noncovalent attachment. The nucleic acid-binding molecule is either a polycationic polymer or a nucleic acid-binding protein: (i) the polycationic polymer is chosen from poly-L-lysine, poly-D-lysine, poly-ethyleneimine, polyamidoamine, polyamine and any free polycations of chemical origin; preferably, the polycationic polymer is poly-L-lysine; (ii) the nucleic acid-binding protein is chosen from histones, protamine, ornithine, putrescine, spermidine, spermine, transcription factors and homeobox proteins; preferably, the nucleic acid-binding protein is a protamine and/or a histone.

During the preparation of the conjugate according to the invention, which comprises a nucleic acid-binding domain such as protamine and/or histones, the binding domain is preferably added in excess. This binding
5 domain is then present in excess in the conjugate. The term "in excess" is intended to denote that the nucleic acid-binding domain and the other components of the conjugate are not present in a stoichiometric amount.

10 The presence in large excess of nucleic acid-binding molecules such as protamine or histones allows and promotes the compacting of the nucleic acid molecule, thus allowing effective transfection of the nucleic acid molecule into the cell and, more particularly,
15 translocation and targeting of the nucleic acid molecule into the nucleus of the cell. Moreover, compacting of the nucleic acid molecule of the invention by a nucleic acid-binding molecule such as protamine or histones makes it possible to protect said
20 nucleic acid molecule against degradations by cellular and extracellular nucleases. The use of protamine and histones for promoting the transfection and expression of a nucleic acid molecule has been known for a long time by those skilled in the art (Wienhues et al.
25 (1987) and Dubes and Wegrzyn (1978)).

The conjugate according to the invention is characterized in that said translocation domain derives from a bacterial or viral toxin, but does not contain
30 the part of the toxin which confers on it its toxic effect. The bacterial or viral toxin is chosen from *Pseudomonas* exotoxin A, diphtheria toxin, cholera toxin, *Bacillus anthrax* toxin, Pertussis toxin, *Shigella* Shiga toxin, Shiga toxin-related toxin,
35 *Escherichia coli* toxins, colicin A, d-endotoxin and *Haemophilus A* hemagglutinin. In a preferred embodiment, the translocation domain is *Pseudomonas aeruginosa* exotoxin A. In another preferred embodiment, the

translocation domain is the nontoxic B fragment of the *Shigella* Shiga toxin.

5 In another preferred embodiment, the translocation domain is a fragment of *Haemophilus* A hemagglutinin. This fragment of influenza A hemagglutinin (HA) may be modified at its C-terminal end by adding a cysteine or by adding a short peptide sequence terminating with a cysteine, in order to make the cysteine react with the
10 coupling agent, which is preferably APDP.

The conjugate according to the invention is characterized in that the antibody is a monoclonal antibody, or a polyclonal antibody, specific for a
15 membrane-bound surface antigen. According to one of the preferred embodiments of the invention, the antibody binds specifically to the G250 antigen characteristic of human renal cell carcinomas (RCCs). According to a preferred embodiment of the invention, the antibody of
20 the invention is the G250 antibody described by Oosterwijk et al. (1986, Int. J. Cancer. 38: 489-494) and which was the subject of international patent application WO 88/08854. According to another preferred embodiment, the antibody according to the present
25 invention is a 5C5 monoclonal antibody obtained with the 5C5 hybridoma deposited at the CNCM under the No. I-2184. The antibody according to the invention is either in the form of a single-chain antibody or in the form of a chimeric antibody or of a humanized antibody.
30 According to a particular embodiment, the antibody is an antibody fragment, preferably an F(ab')₂, Fab' or Fv fragment.

The DNA-antibody conjugate of the present invention may
35 be administered according to various routes known by those skilled in the art. For example, it may be administered intravenously, intraperitoneally, intra-

muscularly, subcutaneously, intratumorally, anally or rectally.

Finally, the invention relates to a conjugate as described above, as a medicinal product. More particularly, the invention relates to a conjugate as described above, as a medicinal product for gene therapy, and more precisely for the treatment of acquired or constitutional genetic diseases. According to the invention, the acquired diseases are selected from the group composed of cancers and infectious diseases. Among the cancers according to the invention, mention may be made of renal cell carcinoma (RCC), melanoma, chronic myeloid leukemia, acute myeloid leukemia, Burkitt's lymphoma, small cell lung cancer, neuroblastoma, retinoblastoma, glioblastoma, hepatocarcinoma, rhabdomyosarcoma, gastric adenocarcinoma, colon carcinoma, ovarian cancer, mammary carcinoma, uterine cancer and testicular carcinoma. Preferably, the invention relates to a conjugate as described above, as a medicinal product for the treatment of renal cell carcinoma (RCC). Among the infectious diseases, mention may preferably be made of AIDS and hepatitis.

According to the invention, the constitutional diseases are preferably selected from the group composed of myopathies, and more particularly Duchenne myopathy (DM), Steinert's myopathy and spinal muscular atrophy (SMA), cystic fibrosis, amyotrophic lateral sclerosis (ALS), hemophilia, hemoglobinopathies, neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's chorea, Gaucher's disease, Lesch-Nyhan disease, immune deficiencies related to a deficiency in adenosine deaminase or in purine nucleoside phosphorylase, pulmonary emphysema and hypercholesterolemia.

It is evident that the compound according to the invention has multiple applications depending on the nature of the DNA sequence selected and on the nature of the antibody selected. These multiple applications
5 can be easily envisaged by those skilled in the art and cannot be mentioned exhaustively.

The invention also relates to a pharmaceutical composition, in particular for the treatment of
10 diseases by gene therapy, which comprises a therapeutically effective amount of a conjugate according to the invention and a pharmaceutically acceptable vehicle.

15 The present invention also relates to a method for transferring a nucleic acid molecule into a cell, characterized in that the conjugate according to the invention is brought into contact with said cell in such a way as to transfect said cell with said
20 conjugate. Preferably, the nucleic acid molecule encodes a protein product of interest which is effectively expressed in said transfected cell.

According to a preferred embodiment of the invention,
25 the nucleic acid molecule is double-stranded DNA encoding a protein product of interest. The present invention therefore provides an effective system which allows the transit of the double-stranded DNA molecule across the cytoplasmic cell membrane, transport to the
30 nucleus, entry into the nucleus and maintenance of this molecule in the functional state, in the nucleus. Persistence of the expression of the protein product encoded by the DNA molecule is obtained either by stable integration of the DNA molecule into the
35 chromosomal DNA of the target cell or by maintaining the DNA molecule in the form of an extrachromosomal replicon. One of the objects of the present invention is therefore to provide a method, characterized in that

said nucleic acid molecule is maintained in the form of an extrachromosomal replicon in said cell. According to another embodiment, the present invention provides a method, characterized in that said nucleic acid molecule integrates into the genomic and/or mitochondrial DNA of said transfected cell.

The cell targeted by the compound of the present invention is a prokaryotic or eukaryotic, animal or plant cell. According to a preferred embodiment, the invention relates to a method, characterized in that said cell is a eukaryotic cell, preferably a mammalian cell, and preferably a human cell.

Finally, the invention relates to the cells transfected with the conjugate according to the invention, the cell preferably being a eukaryotic cell, more particularly a mammalian cell, and preferably a human cell.

Other characteristics and advantages of the present invention will be more clearly demonstrated upon reading the following examples.

In these example, reference will be made to the following figures:

Figure No. 1: Production of murine interleukin 2 by RCC lines antinfected with the conjugate G250/BZQ/Il2 (G250=DNA) in the presence or absence of exotoxin A.

Figure No. 2: Production of murine interleukin 2 by RCC lines antinfected with the conjugates biotinylated G250/avidin/BZQ/PL/Il2 (G250AvPL) and biotinylated (G250+ExoT)/avidin/BZQ/PL/Il2 (G250AvPLTox); negative control: avidin/BZQ/PL/Il2 (AvPL).

Figure 3: Expression of the CD4 molecule at the surface of human kidney cancer cells after antifection *in vitro* (% positive cells).

Figure 4: Measurement of mouse IL-2 secretion 11 days after antifection of human kidney cancer cells *in vitro*.

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Figure 5: Induction of human kidney [lacuna] cell death after antifection *in vitro* with the human Bax cDNA.

Figure 6: Measurement of the volume of tumors obtained on days D=7 and D=19 after tumor transplantation, after antifection of the murine Bax cDNA *in vivo*, with an injection (30 µg of DNA) on day D=7.

Figure 7: Infiltration of tumors with CD16+ cells after antifection of the murine Bax cDNA.

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EXAMPLES

EXAMPLE 1: MATERIALS AND METHODS (see Dürrbach *et al.*,

20 The antibody-mediated endocytosis of G250 tumor-associated antigen allows targeted gene transfer to human renal-cell-carcinoma *in vitro*, Cancer Gene Therapy, In Press)

25 **1.1 Cells**

The renal carcinoma cell lines used are: IGR/RCC-17, (HIEG), IGR/RCC-40 (ROB), IGR/RCC-47 (FRAP), IGR/RCC-58 (MOJ), which derive from three primary tumors (-17, -40 and -47) and from one adrenal metastasis (-58), from
30 four patients suffering from RCC at the metastatic stage. According to histological criteria, RCC-17, -40 and -58 correspond to clear cell carcinomas and RCC-47 to a particular form of clear cell carcinoma with typical papillary foci which are highly tumorigenic in
35 SCID mice (Angevin *et al.* (1997) *Process. Am. Asso. Cancer Res.* 38: 238; Goulkhova *et al.* (1998) *Genes Chrom. Cancer* 22: 171-178). The establishment of the culture *in vitro* and also the characterization of the

RCC cell lines were carried out as previously described (Angevin et al. 1997 Int. J. Cancer 72: 434-440). The cells are cultured at 37°C in an atmosphere comprising 5% CO₂, in Dulbecco's modified MEM medium with Glutamax-1 (Gibco BRL, Paisley, Scotland) supplemented with 10% of fetal calf serum (Seromed, Berlin, Germany), 5% of nonessential amino acids, 10 mM of sodium pyruvate (Gibco BRL) and a mixture of penicillin/streptomycin (10 mg/ml) (Seromed).

10

1.2. Immunophenotyping of the G250 antigen

Expression of the G250 antigen associated with RCC tumors was directly tested by indirect immunolabeling using the mouse IgG1 monoclonal antibody G250 (G250 mAb) described previously (Oosterwijk et al., 1986, Int. J. Cancer 38: 489-494). A suspension of 5×10^5 cells, obtained by trypsinization, was washed twice in RCC culture medium; the cells are then incubated with the G250 mAb, washed 3 times in PBS (phosphate-buffered saline) and then incubated with an FITC-labeled F(ab')₂ fragment of a goat anti-mouse IgG antibody. The NKTA monoclonal antibody having the same isotype (IgG1 directed against a clonotypic determinant of the TCR α/β) (kindly provided by Dr Thierry Hercend, France) was used as a negative control. Flow cytometry was carried with a FACScan cytometer (Becton-Dickinson, Sunnyvale, CA, USA) using the Cellquest program.

1.3. Endocytosis experiments

The G250 antibody and iron-loaded human apo-transferrin (Sigma, St Louis, MO, USA) were coupled, respectively, with fluoresceine isothiocyanate (Sigma) and with lissamine Rhodamine B sulfonyl chloride as described previously (Maxfield et al., 1978, Cell 14: 805-810; Brandzaeg, 1973, Scan. J. Immunol. 2: 273-290). The conjugated proteins are separated from the free fluorochromes by gel filtration on a Sephadex G50 column (Pharmacia, Uppsala, Sweden). Specific binding

35

of the coupled proteins with the cell surface receptors was determined by competition experiments using a 100-fold higher concentration of noncoupled proteins. The plasmid DNA BMGneo-mIL2 containing the mouse interleukin 2 (IL-2) cDNA under the control of the inducible promoter of the metallothionein gene (Karasuyama and Melchers, 1988, Eur. J. Immunol. 18: 97-104) (1 mg/ml) is incubated (vol/vol) with EZ-link-Biotin-LC-ASA reconstituted in ethanol (2 mg/ml) (Pierce, Rockford, IL USA) and exposed to UV radiation (365 nm) for 15 min at 4°C. The plasmid DNA is then precipitated with ethanol (final concentration 70%) for 30 min at -20°C. The labeling efficiency is determined using an ELISA assay on microplates coated with poly-L-lysine, using streptavidin-conjugated alkaline phosphatase.

In order to test endocytosis, cells cultured for two days on cover slips are washed three times with RPMI-1640 (Gibco BRL) containing 1 mg/ml of bovine serum albumin (BSA) and are then incubated twice for 15 min in RPMI-1640 containing 1 mg/ml of BSA at 37°C, with or without cytochalasin D (5 M) (Sigma). The cells are then incubated for one hour at 4°C with rhodamine-conjugated transferrin (50 nM) and FITC-labeled G250 monoclonal antibody in RPMI-1640 containing 1 mg/ml of BSA, with or without cytochalasin D (5 M), and then transferred to 37°C for varying times with rhodamine-transferrin only (pulse) or with the FITC-labeled G250 mAb. The cells are washed three times with cold PBS, fixed for 20 min with a solution of 4% para-formaldehyde, 0.025% glutaraldehyde in PBS at 4°C and prepared for the epifluorescence analysis. In order to analyze the distribution of the G250 mAb-plasmid conjugate by double labeling, the cells were incubated continuously as described above, either with FITC-labeled G250 mAb conjugated with biotinylated plasmid

DNA, or with a mixture of FITC-labeled G250 mAb and biotinylated plasmid DNA, as a control.

After fixing, the cells were washed twice in PBS, 5 incubated for 10 min with 0.1% of sodium borohydrate in PBS (ICN, Costa Mesa, CA, USA) and then for 10 min with ammonium chloride (50 mM in PBS) (Sigma). Depending on the experimental conditions, the cells are either directly analyzed by immunofluorescence in order to 10 detect the FITC-labeled G250 mAb, or permeabilized with PBS containing 0.05% of saponin or 0.1% of Triton X100 (ICN) and then labeled with Texas-red-conjugated streptavidin (20 mg/ml) (Pierce). The actin filaments are labeled with rhodamine-phalloidin, according to the 15 manufacturer's recommendations (Sigma).

The cells are then visualized with an Axiophot microscope (Zeiss, Oberkochen, Germany).

20 **1.4. Antifection and analysis of the heterologous expression**

For transfection, 3×10^5 freshly trypsinized RCC cells are incubated for 30 min at 4°C with different concentrations of mAb-DNA conjugates according to the 25 invention, in 1 ml of RPMI-1640 without serum. The cells are then incubated for 4 hours at 37°C in 1 ml of RPMI-1640 without serum, containing 4×10^5 M of chloroquine (Sigma) and finally resuspended in 2 ml of DMEM supplemented with glutamax-1 (Gibco BRL) and 10% 30 of fetal calf serum. In separate experiments, the RCC cells are incubated with the mouse IL-2 cDNA conjugated to the G250 mAb, in the presence of cytochalasin D, for 1 hour at 4°C and 4 hours at 37°C. The conjugates still bound to the cell surface were detached with a solution 35 of RPMI-1640, pH 2.2, containing 0.1 M glycine for 2 min at 4°C. Two volumes of RPMI-1640, pH 9.0, are then added for 3 min and the cells are incubated in a normal culture medium. In order to determine the

production of murine interleukin 2, 100 µl of cell culture supernatants were removed on different days after transfection. The cytokine production in the medium was determined using the mouse IL-2-specific DuoSet ELISA kit (ref. 80-3573-00) (15 pg/ml detection threshold) (Genzyme Diagnostics, Cambridge, MA, USA).

EXAMPLE 2: Conjugates G250/BZQ/Il2 and G250/BZQ/Il2+ExoT

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2.1 Preparation of the conjugate G250/BZQ/Il2

The conjugate G250/BZQ/Il2 is prepared by coupling between the G250 monoclonal antibody and a plasmid encoding murine interleukin 2 (mIl-2), by means of benzoquinone (BZQ), according to the coupling method previously described by Poncet et al. (1996, Gene Therapy 3: 731-738).

The BZQ dissolved in absolute ethanol at a concentration of 30 mg/ml is added to a solution of purified monoclonal antibody dissolved in PBS at a concentration of at least 2 mg/ml, so as to give a final solution containing 3 mg/ml of BZQ. One tenth of the final volume is then added in the form of 1M potassium phosphate buffer, pH 6.0. After 90 min at room temperature, in the dark, the activated monoclonal antibody is separated from the excess BZQ by chromatography on a G25M column (Pharmacia) presaturated with 1% BSA in 0.15M NaCl, collected and then mixed with the purified plasmid DNA (10 times the amount of antibody). The solution is mixed with 0.1 M of carbonate buffer, pH 8.7, and incubated for 48 hours at 4°C. The mAb-DNA conjugate is concentrated by gel filtration on a Superose 6HR FPLC column (Pharmacia) in order to remove the excesses of free antibody likely to compete with the DNA-antibody conjugate. The fractions collected are dialyzed against PBS and concentrated using a Centricon 10 cartridge (Amicon, MA, USA). The

amounts of purified soluble conjugates are expressed as the amount of plasmid DNA initially used in the reaction.

5 **2.2. Antifection of RCC lines with the conjugates G250/BZQ/Il2 and G250/BZQ/Il2+ExoT**

We compared the Il-2 measurement after transferring this conjugate into RCC lines, after adding, or not adding, *Pseudomonas Aeruginosa* exotoxin A (ExoT) to the
10 culture medium. The exotoxin A sold by Sigma is added to the conjugate G250/BZQ/Il2.

The conjugates G250/BZQ/Il2 and G250/BZQ/Il2+ExoT are brought into contact with 10^5 RCC line cells in culture
15 in a serum-free medium for 4 hours at 37°C according to the protocol previously described. The cells are put back into culture in normal medium after washing. The production of Il-2 is measured 10 days later using the DuoSet ELISA kit (ref. 80-3573-00 Genzyme Diagnostics).

20 The cells antifectioned with conjugate G250/BZQ/Il2+ExoT produce approximately 3 times more murine Il-2 (371 pg/ 10^6 cells) than the cells antifectioned with the conjugate G250/BZQ/Il2 (165 pg/ 10^6 cells) (Figure
25 No. 1).

EXAMPLE 3: Conjugates biotinylated G250/avidin/-BZQ/PL/Il2 and biotinylated (G250+ExoT)/avidin/-BZQ/PL/Il2

30

3.1 Preparation of the conjugates

The central body of the conjugates biotinylated G250/-avidin/BZQ/PL/Il2 and biotinylated (G250+ExoT)/avidin/-BZQ/PL/Il2 consists of a tetravalent avidin (Av)
35 molecule which is, initially, activated with benzoquinone according to the protocol described above. The activated avidin binds the poly-L-lysine molecules which are molecules with a high affinity for DNA. The

avidin/BZQ/PL complex is brought into contact with the plasmid encoding mouse interleukin 2 (Il-2). The complex is then associated with the G250 monoclonal antibody and/or with the exotoxin A (ExoT), both
5 biotinylated beforehand.

3.2 Antifection of RCC lines with the conjugates biotinylated G250/avidin/BZQ/PL/Il2 and biotinylated (G250+ExoT)/avidin/BZQ/PL/Il2

10 The various complexes are brought into contact with 10^5 RCC line cells in culture in a serum-free medium for 4 hours at 37°C according to the protocol described above. The cells are put back into culture in normal medium after washing. The production of Il-2 is
15 measured 10 days later, using the DuoSet ELISA kit (ref. 80-3573-00, Genzyme Diagnostics).

The results are given in figure No. 2. In the control experiment in which the G250 monoclonal antibody was
20 omitted, some production of mIl-2 is measured ($127 \text{ pg}/10^6$ cells, AvPL), certainly due to the nonspecific attachment of the poly-L-lysine and/or avidin molecules to the surface of the cells. Adding G250 mAb to the avidin/BZQ/PL/Il2 complex increases by
25 2-fold the production of murine interleukin 2 ($261 \text{ pg}/10^6$ cells instead of $127 \text{ pg}/10^6$ cells); the additional presence of exotoxin A (ExoT) makes it possible to increase by 10-fold the production of mIl-2 ($1347 \text{ pg}/10^6$ cells) (figure No. 2).

30

EXAMPLE 4: Conjugate biotinylated G250/neutravidin/-biotinylated histone H1/biotinylated *influenzae* hemagglutinin (HA) fusogenic peptide/CD4

The various complexes as mentioned on the figures are
35 brought into contact with RCCs, as described in example 3, section 3.2.

Figure 3 represents the analysis by flow cytometry of the human RCC cells bearing the G250 Ag, collected 7 days after antifection of the cDNA encoding the human CD4 molecule and labeled with a human anti-CD4 mAb. In this way, approximately 20% of the cells express this molecule. The vector used comprised all of the molecules, G250, H1, HA, cDNA. The sequence of the HA peptide used is as follows:

GLFEAIAGFIENGWEGMIDGGGCGSGSYTDIEMNRLGKG.

EXAMPLE 5: Conjugate biotinylated G250/neutravidin/-biotinylated histone H1/IL2 and conjugate biotinylated G250/neutravidin/biotinylated histone H1/biotinylated HA fusogenic peptide/IL2

Figure 4 represents the result of an antifection of human RCC cells bearing the G250 Ag, collected 11 days after antifection of the cDNA encoding mouse interleukin-2. The amount of IL-2 secreted by the RCCs brought into contact with the cDNA on its own or coupled to neutravidin is 80 pg/10⁶ cells, the amount of IL-2 secreted by the RCCs brought into contact with a conjugate comprising G250/H1/cDNA is 1200 pg/10⁶ cells, and the amount of IL-2 secreted by the RCCs brought into contact with a conjugate comprising G250/H1/HA/cDNA is 3100 pg/10⁶ cells.

EXAMPLE 6: Biotinylated G250/neutravidin/biotinylated histone H1/BAX and biotinylated G250/neutravidin/-biotinylated H1 histone/biotinylated HA fusogenic peptide/BAX

Figure 5 represents the result of an antifection of human RCC cells bearing the G250 Ag, collected 11 days after antifection of the cDNA encoding the human Bax pro-apoptotic molecule. Cell death was assessed by Trypan blue staining. The control cDNA used corresponds to the *green fluorescent protein* (GFP) gene. It is possible to note a considerable increase in the loss of viability linked to the attachment of the G250 mAb,

"vector BAX without HA", which is increased by attaching the HA fusogenic peptide, "vector BAX with HA".

5 **EXAMPLE 7: Antifection in vivo with the conjugate biotinylated 5C5/neutravidin/biotinylated histone H1/biotinylated HA fusogenic peptide/murine BAX**

7.1. Protocol

10 Various conjugates are prepared using:

- 10 µg of 5C5 antibody
- 30 µg of murine BAX DNA
- 15 µg of histone H1
- 0.5 µg of HA peptide
- 15 - 4 µg of neutravidin.

An RCC tumor was transplanted, subcutaneously into nine irradiated nude mice, which act as a control, on day D=0. These transplanted mice have no conjugate.

20

An RCC tumor was transplanted, subcutaneously into ten irradiated nude mice on day D=0 and then on day D=7; they received the conjugate neutravidin/HA/H1/Bax intravenously in a single injection.

25

An RCC tumor was transplanted, subcutaneously into ten irradiated nude mice on day D=0 and then on day D=7; they received the conjugate 5C5/neutravidin/HA/H1/Bax intravenously in a single injection.

30

The size of the tumors was then evaluated on day D5, D8, D12 and D19 after injection. The mice were sacrificed on day D19.

35 **7.2 Results**

A decrease in tumor growth, less than the growth noted in the control groups, is observed in 6 out of 10 mice receiving the whole complex and 1 out 10 in the group

treated with the complex without antibody, this being on day 19 after the first injection (figure No. 6).

5 Interestingly, the labeling found in the tumors
antifected with Bax, using a fluorescent mAb directed
against the mouse CD16 molecule present on natural
killer cells (NK cells), macrophages and granulocytes,
clearly indicates the possibility of recruiting
effector cells which can contribute fully to the
10 antitumor response (figure No. 7).

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